

# Detecting the Effects of Toxic Agents on Spermatogenesis Using DNA Probes

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Advances in the molecular biology of spermatogenesis suggest that DNA probes can be used to monitor the effects of toxic agents in male germ cells of mammals. Molecular hybridization analyses with DNA probes can provide a reproducible methodology capable of detecting changes ranging from massive deletions to single base pair substitutions in the genome of exposed individuals. A constantly increasing number of DNA probes that can be used to detect such alterations in human sperm DNA exist for both ubiquitously expressed proteins and for genes solely expressed in the testis. In this chapter, the currently available testicular stage-specific and/or cell type-specific DNA probes and the techniques by which they can be utilized in reproductive toxicology studies are discussed. The advantages, limitations, and future technological advances of this novel biological marker system for the human male reproductive system are also considered.

## Introduction

During the process of spermatogenesis, diploid stem cells differentiate first into functionally tetraploid cells during meiosis and subsequently into haploid spermatozoa. Following a series of stem cell divisions, primary spermatocytes undergo meiosis, the interval in which chromosomes synapse and genetic recombination occurs. This phase of male germ cell development is followed by two meiotic divisions that produce the haploid round spermatid, a cell type that transforms into the spermatozoon during spermiogenesis, the haploid phase of the spermatogenic cycle. Our precise knowledge of the morphological events of spermatogenesis, combined with established testicular cell separation techniques, has made the testis a most amenable organ to explore biochemically.

The application of molecular biology to the study of mammalian testicular differentiation is providing investigators with insights into many of the molecular mechanisms that regulate male germ cell formation. Gene expression during spermatogenesis is temporally and spatially regulated with precision with a large number of macromolecules and organelles being synthesized in specific cell types during the lengthy continuum of testicular cell differentiation (1). Although variants of ubiquitous enzymes and structural proteins are expressed in many organs, the testis appears to be an especially rich source of isozymes (2). Presumably because of the specialized requirements essential to produce a spermatozoon, a number of unique testicular isozymes coding for proteins such as lactate dehydrogen-

ase, phosphoglycerate kinase, and cytochrome *c* have evolved (Fig. 1) (1,2). In addition to the testicular isozymes, a large number of male-specific structural proteins of the maturing spermatid and the spermatozoon have been identified (Fig. 1) (1). Biochemical analyses of the differentiating haploid germ cells that transform into the highly polarized spermatozoon suggest we have only begun to define the many possible sperm-specific molecular markers. As a result of such studies and the well-characterized sequence of events leading to the formation of spermatozoa, efforts to monitor the effects of toxic agents on male germ cells can use the substantial knowledge base and the numerous DNA probes that are currently available for the mammalian testis.

In this chapter, I shall discuss how present day recombinant DNA techniques can be used to measure the effect of toxic agents on testicular cells. I shall first describe some of the growing number of DNA probes for the mammalian genome that are available and then discuss several general approaches through which the DNA probes can be used to screen for changes in male germ cell DNA following exposure to toxic substances. I shall especially focus my discussion on DNA markers specific to testicular cell types or stages of spermatogenesis that will provide a means to dissect the site and define the mechanisms of action of toxic agents in the mammalian testis. In addition to providing a summary of DNA probes that now exist, it is hoped that this report will stimulate development of improved DNA monitoring procedures for compounds producing deleterious effects on male germ cells and lead in the near future to the use of DNA probes as biological markers for toxic agents and for risk assessment.

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## MOUSE SPERMATOGENESIS

CELL TYPE	SPERMATOGONIA	SPERMATOCYTES I (Pachytene)	II (Round → Elongating)	SPERM
Ploidy	2N	4N	2N	N
Rate of Differentiation (days)	6	11	19	20
DNA Synthesis	—	—	—	—
mRNA Synthesis	—	—	—	—
Cyto. RNA (pg/cell)		9.1	2.2	0.9
Poly (A) <sup>+</sup> RNA (ng/μg)		6	11	3
Protein Synthesis				
Histones	—	—	—	—
PGK-2	—	—	—	—
Protamines				
MP1			—	—
MP2			—	—
TP			—	?
LDH-X		—	—	—
Acrosin		—	—	—
β Actin (2.1 kB)	—	—	—	—
γ Actin (2.1 kB)	—	—	—	—
α Actin (?) (1.5 kB)	—	—	—	—
c-abl (6.2 and 8.0 kB)	—	—	—	—
c-abl (4.7 kB)	—	—	—	—

FIGURE 1. Gene expression during spermatogenesis. This chart represents a summary of the temporal expression of a few representative mouse testicular proteins discussed in a recent review of gene expression during spermatogenesis (1). Abbreviations: PGK-2, phosphoglycerate kinase 2; MP, mouse protamine; TP, testis-protein; LDH-X, lactate dehydrogenase C4. A solid line indicates the approximate interval when a protein is synthesized. A dashed line represents the interval when mRNA for a protein has been detected but the protein appears not to be yet synthesized; i.e., the interval when the mRNA is stored as a ribonucleoprotein. An open box indicates the interval when mp2 is synthesized as a precursor and subsequently processed to the mature form of the P2 protamine. Question marks represent the author's uncertainty as to the exact time when TP synthesis terminates and to the identity of the 1.5 kB actin mRNA.

## Overview of General Use of DNA Probes

Molecular hybridization analysis with DNA probes offers a superior, highly reproducible means to identify subtle alterations in DNA sequence. In practice, a fragment of DNA or RNA of known sequence is compared to an equivalent experimental DNA fragment by annealing the two and monitoring for base-pair mismatches. With appropriate controls, differences in DNA sequence ranging from chromosome deletions and translocations to single base-pair substitutions between the control probe and the experimental DNA can be detected. Such DNA alterations would reflect changes induced in the genome by exposure to a toxic agent. These hybridization procedures are predicated upon our ability to obtain defined wild-type genes or sequences by

standard cloning procedures. These DNA probes would then serve as reference sequences. When specific defined probes are used, it will be possible to correlate DNA changes with knowledge of the gene structure of known probes. Nonspecific probes to noncoding or undefined regions of the genome would assay for general changes in the DNA. Such procedures would, however, be confined to monitoring identical changes in the DNA of a large number of the cells examined. Methods to analyze the DNA from single cells are badly needed since fluorescence-activated cell sorters can readily provide single cell samples for analysis. Although major chromosomal alterations such as deletions in single cells can be detected by *in situ* hybridization, to detect subtle changes, amplification methods that increase the number of copies of a DNA fragment up to 10<sup>6</sup>-fold must be employed. Unfortunately, the amplification procedures

in current use introduce errors at high frequencies. Amplification procedures which introduce low frequencies of error remain to be developed.

To detect lesions in DNA in the male reproductive tract, DNA can be prepared from ejaculated spermatozoa (3). The sequence of a particular gene or group of genes would be compared to the wild-type sequence of a well-characterized DNA probe by one or more of the hybridization procedures described later in the chapter. Such an approach would be capable of detecting DNA changes that are neither corrected by the male germ cell repair mechanisms nor are of a sufficiently disastrous nature that they prevent spermatogenesis from proceeding to completion. For the latter case, cytological examination of ejaculates (if any sperm are produced) and/or testicular biopsies would be required.

Any defined region of the genome for which a DNA probe is available can be monitored. Since toxic agents for the most part are assumed to act randomly on DNA, to apply DNA probes to human studies, a large group of DNA probes homologous to as much of the human genome as possible should be used. The greater the number of sequences monitored, the more likely one will successfully detect an alteration. The present limitation that only a small part of the total genome can be monitored at any one time is a serious shortcoming of such a screening procedure. Despite this constraint, the application of hybridization techniques with a sizeable group of DNA probes offers one promising way to start using a molecular approach to detect DNA damage. Considering the rapidly evolving advances in molecular biology, it is likely that the technical problem of surveillance of large regions of the genome of single human cells will not be insurmountable.

DNA probes that could be used to detect genomic alterations in the DNA from human sperm fall into two categories: probes for ubiquitously expressed proteins such as the globins or dihydrofolate reductase, and probes for genes solely expressed in the testis. The former class of DNA probe offers an easily obtained and constantly increasing source of biological marker that can be used to monitor random damage to the human genome. The latter class of stage- or cell-type-specific testicular DNA probe could be used to screen random alterations in a defined part of the human genome. It would have the additional advantage that, when applied to an animal model system, the effect of the toxic agent could be related to specific time periods of testicular cell differentiation, assuming the production of a specific testicular gene product has been disrupted. Such complementary human and animal studies would provide a useful response comparison between mammals and allow the mechanism(s) of action of the compound in question to be investigated. Moreover, the availability of DNA probes for testicular genes allows investigation of possible genetic and/or cell type "hot spots" (regions of the genome highly susceptible to damage) during germ cell differentiation. For these reasons, the next section of this chapter will describe many of the testis-specific

DNA probes currently being used by investigators (Table 1).

## Testicular-Stage-Specific and Cell-Type-Specific DNA Probes

To date, most attempts of DNA cloning during spermatogenesis have been directed toward two intervals of spermatogenesis, meiosis and spermiogenesis (4-6). This is because the meiotic pachytene spermatocytes and the haploid round spermatids represent two critical time periods for gene expression during male germ cell development. Moreover, highly enriched populations of pachytene spermatocytes and round spermatids can be readily obtained by cell separation techniques such as the Staput unit gravity procedure or Percoll density gradient centrifugation because of their marked size differences and abundance in the sexually mature testis (1,7,8). Much progress has also been made in obtaining molecular markers for cells such as Sertoli cells, because when isolated from prepubertal animals, this type of testicular somatic cell can be easily cultured (9). The testis offers an excellent system to study gene regulation during differentiation and improvements in the ability to isolate highly enriched populations of numerically minor testicular cell types such as the type A and B spermatogonia are expected. The growing awareness of these opportunities by molecular biologists suggests that unique DNA probes for many nonabundant testicular cell types will soon become available.

To date, the vast majority of DNA probes for testicular genes have been isolated from animal systems such as the mouse, rat, or bull. The origin of these probes will not be a problem for application to human DNA. Since DNA probes for homologous genes in humans and other vertebrates share substantial sequence homology, the animal probes can be used to isolate equivalent human sequences from human cDNA or genomic DNA libraries. Investigators with long-term, committed in-

Table 1. Testicular DNA probes.

Type of probe	Probe for
Meiotic	Lactate dehydrogenase C4 (14) Phosphoglycerate kinase 2 (17) Cytochrome <i>c</i> <sub>1</sub> Histone variants (21)
Postmeiotic	Protamine 1 and 2 (22,23) Testis protein (TP) (29,32) Actin variant (34) Tubulin variants (36,54)
Leydig cell	Pro-opiomelanocortin (40) Renin (41)
Sertoli cell	Follicle-stimulating hormone (42) Androgen-binding protein (43) Ceruloplasmin (42) Transferrin (42) Sulfated glycoproteins (42) Inhibin (44) Somatomedin Mullerian-inhibiting substance (45)

terests in human toxicology studies should seriously consider the direct isolation and characterization of human DNA probes, as human testicular cDNA libraries and human genomic DNA libraries are available. For cases where both rodent and human probes are available, the animal studies can be used to validate the DNA probe marker for human use, and more importantly, the animal studies can be used to reveal the basic mechanism of action by which specific chemicals interact with the genome.

## Meiotic DNA Probes

**Lactate Dehydrogenase C4.** Lactate dehydrogenase C4 (LDH X) is one of the best-characterized proteins in the testis (2). It appears to be testis-specific and is synthesized initially during meiosis and in decreasing amounts during early spermiogenesis (10). Since LDH C4 is a relatively abundant testicular enzyme, LDH C4 mRNA represents as much as 0.18% of total functional mRNA in mouse pachytene spermatocytes (11). Because LDH C4 is also present on the surface of mature spermatozoa, it has been used extensively in immunocontraceptive efforts (12). Although the protein sequence of LDH C4 from rodents has been known for some time (13), only recently has a cDNA probe for human LDH C4 been isolated (14).

**Phosphoglycerate Kinase 2.** Two distinct forms of phosphoglycerate kinase (PGK) have been characterized in mammals. PGK-1 is an X-linked gene that is expressed in somatic cells, whereas the autosomally derived isozyme, PGK-2, is specific to the testis (15). Although the PGK-2 protein is synthesized during spermiogenesis, the gene appears to be initially transcribed during meiosis with increased synthesis of PGK-2 mRNA in spermatids (16). Recent elegant studies of the human PGK multigene family have produced detailed sequence knowledge and DNA probes for these important enzymes (17).

**Cytochrome  $c_t$ .** Cytochrome  $c$ , the electron transport protein from the mitochondrial respiratory chain, exists in two forms in the testis (18). One variant, cytochrome  $c_t$ , is restricted to the testis, while the other somatic variant, cytochrome  $c_s$ , is presumably found in all tissues. Indirect immunofluorescence with monospecific antibodies first detects cytochrome  $c_t$  in the mitochondria of pachytene spermatocytes and in later stages of spermatogenesis, whereas cytochrome  $c_s$  is found in the mitochondria of interstitial cells, Leydig cells, and spermatogonia. Sequence analysis of the two mouse cytochrome  $c$  molecules has revealed that cytochrome  $c_t$  differs from the cytochrome  $c_s$  in 13 amino acid residues (19). Although at this writing DNA probes only exist for the somatic variant of cytochrome  $c$ , the known sequence for the testicular form of cytochrome  $c$  would allow appropriate oligonucleotide DNA probes to be prepared. Such oligonucleotides would facilitate the isolation of cDNA or genomic DNA probes for human cytochrome  $c_t$ .

**Meiotic Histone Variants.** During spermatogene-

sis, a dramatic reorganization of the germ cell nucleus occurs. This transformation, ultimately producing a sperm nucleus with highly compacted DNA, is accompanied by the replacement of histones with a group of transiently associated nuclear proteins and finally with protamine (20). In addition to the standard complement of histone molecules found in mammalian cells, the meiotic pachytene spermatocyte contains several additional histone variants believed to be unique to the testis. DNA sequence analysis of one testis-specific histone variant, Hlt, reveals it to be a unique gene product and not a posttranslational modification of an existing histone (21). The availability of a specific DNA probe for one of these rat meiotic histones provides a means to obtain the equivalent probe for the human male meiotic histone gene. Probes for other testis-specific histones can also be isolated.

Although currently few in number, these DNA probes coding for well-characterized and essential meiotic proteins provide a start in building a battery of DNA probes to monitor gene expression during this critical interval of spermatogenesis.

## Postmeiotic DNA Probes

**Protamines.** The predominant proteins in mammalian spermatozoa are the protamines, a group of small arginine-rich DNA-binding proteins that aid in nuclear DNA compaction during spermiogenesis (20). With the exception of the mouse and human, most mammalian spermatozoa have been reported to contain one type of protamine. In the mouse, two protamine variants, mP1 and mP2, have been identified by DNA sequence analysis of isolated cDNA clones (22,23). Protein sequence studies have identified similar P1 and P2 human protamine variants (24-26). Although the P1 and P2 protamines differ substantially in size and sequence, in the mouse they are closely linked on chromosome 16 and are temporally and translationally regulated during spermiogenesis (27). It is likely that the human protamine genes are also chromosomally linked. Recently the P1 human protamine has been demonstrated to be located on human chromosome 16 (Reeves and Hecht, unpublished observation).

Northern blots of RNA isolated from prepuberal testes and from isolated meiotic and postmeiotic testicular cell types reveal that the protamines are solely expressed during the haploid interval of spermatogenesis (4,28,29). Moreover, changes in length of protamine mRNAs during spermatogenesis allow the protamine probes to be used as molecular markers to evaluate the extent of spermiogenesis in wild-type, mutant, or chemically induced sterile animals (28). For instance, using the mP1-cDNA probe, no mP1-mRNA is detected in testicular extracts of prepuberal mice up to 20 days of age (a time when spermatogenesis has advanced to meiosis), whereas a 580 nucleotide form of mP1-mRNA is present in the testes of 22-day-old mice (early spermatids are present by day 22), and a heterogeneous population of 580 and 450 nucleotide long mP1-mRNAs are

present in the testes of sexually mature animals (Fig. 2) (29). Cell separation studies confirm that no mP1-mRNA is present in pachytene spermatocytes, a 580 nucleotide mP1-mRNA is found in round spermatids, and elongating spermatids contain an additional 450 nucleotide length mRNA (28). These mRNA length changes are a result of a partial deadenylation of the protamine mRNAs when they move from the ribonucleoprotein particle fraction of the cytoplasm (in round spermatids) onto polysomes (in elongating spermatids). Similar size changes occur for the P2 mouse protamine and for P1 and P2 rat and hamster protamine mRNAs (30).

To test the predictive value of the protamine cDNAs as markers for testicular germ cell differentiation, three mouse mutants—quaking, blind sterile, and testicular feminization—have been examined (31). Quaking (*qk*) is an autosomal mutation causing reduced myelination of the central nervous system and virtual azoospermia in the homozygous male. Cytological examination reveals that the sterility in the male is caused by defects in spermatid differentiation affecting sperm head shaping and tail assembly. Northern blots of testicular RNA from homozygous males demonstrate that both the

450 and 580 nucleotide mP1-mRNAs are present in the testis. This indicates that a substantial number of spermatids have differentiated to at least step 12 of spermiogenesis, a result in agreement with the morphological analysis of the mutant testis.

Blind sterile (*bs*) is an autosomal mutation producing cataracts in both sexes and sterility in the male. Analysis of testicular RNA from *bs/bs* males reveals a large decrease in amount of both size classes of mP1-mRNA, a result in agreement with the cytological report of markedly reduced numbers of haploid cell types in the mutant testis.

Testicular feminization (*Tfm*) is an X-linked mutation where chromosomally male mice fail to respond to testosterone and have only vestigial testis. No mP1-mRNA is detected in extracts of testes of *Tfm/Y* mice, an observation supported by the lack of differentiation of postmeiotic germ cells.

The protamine genes appear to be excellent candidates to serve as probes to monitor genomic defects induced during spermiogenesis. They represent two single copy genes that express abundant postmeiotic testicular mRNAs essential for sperm function. Moreover, the cDNA probes show much homology to the DNA and RNA of many other vertebrates including man. Several laboratories are presently seeking to isolate human probes for these male-specific DNA-binding proteins.

**Testis Protein (TP).** In mammals, histones are not directly replaced by protamines but by a presumably heterogeneous group of basic proteins called testis-specific proteins (TP) (20). TPs are associated with the spermatid nucleus during its transition from its nucleosome-like structure to the smooth, branching fibril of the spermatozoan nucleus. The TPs are subsequently replaced by protamine during spermiogenesis. Recently, cDNA probes for the mouse and rat TPs have been identified (29,32). Phylogenetic studies indicate a strong sequence conservation of TPs in rodents and man. The identical pattern of expression of mouse TP and protamine suggests that these three genes are temporally regulated in the postmeiotic testicular cells and could be used together to monitor postmeiotic gene expression.

**Postmeiotic Actin.** In mammals, actin is encoded by a multigene family that expresses at least six distinct but closely related forms of actin. In addition to the general role actin plays in cell motility and division, secretion, organelle movement, and maintenance of cellular cytoarchitecture, testicular actins are likely to be involved in chromosome movement during meiosis, in shaping specific nuclear structures during spermiogenesis, and in spermatozoan function (33). Although the mRNAs that code for the cytoplasmic  $\beta$ - and  $\gamma$ -actin isotypes have been detected in all testicular cell types throughout spermatogenesis, mRNA encoding an additional actin variant is first detected during spermiogenesis (34). It should be possible to obtain several distinct DNA probes to isotypes of actin that are expressed constitutively during spermatogenesis and to

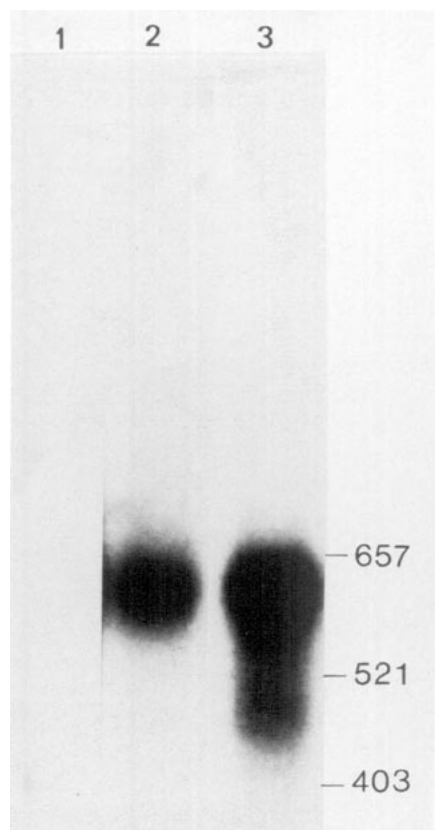


FIGURE 2. Size of protamine 1 mRNA in testicular extracts of prepubertal and sexually mature mice. Ten  $\mu$ g aliquots of purified testicular RNA from CD-1 mice were denatured with glyoxal, electrophoresed in a 2% agarose gel, transferred to nitrocellulose filters and hybridized to  $^{32}$ P-labeled mP1-cDNA. Lane 1: RNA from 16-day-old animals; lane 2: RNA from 24-day-old animals; lane 3: RNA from sexually mature animals. The numbers to the right are nucleotide size markers. From Hecht et al. (31).

other actin isotypes that are temporally expressed in specific stages or cell types.

**Tubulins.** Microtubules consist of heterodimers of  $\alpha$ - and  $\beta$ -tubulin. The  $\alpha$ - and  $\beta$ -tubulin subunits represent distinct sequences, each encoded by multigene families. In the testis, the tubulins are involved in mitotic and meiotic divisions, in changes in cell shape and structure, in the species-specific shaping of the sperm nucleus, and in the synthesis of the axoneme of sperm tails. Protein gel electrophoresis and DNA cloning studies suggest that during spermatogenesis multiple isoforms of  $\alpha$ - and  $\beta$ -tubulin are expressed (33). One form of  $\alpha$ -tubulin encoding a mRNA of 2.1 kB is first detected in postmeiotic cell types (35). Sequence analysis of a mouse testicular  $\alpha$ -tubulin cDNA reveals that the polypeptide it codes for differs from a conserved somatic tubulin sequence in 20% of its amino acids (36). The availability of cDNAs for a number of mouse testicular  $\alpha$ -tubulins provides a set of useful probes to monitor the differential expression of several different cytoplasmic structural genes during testicular germ cell development. A detailed study of the expression of the  $\beta$ -tubulin multigene family during spermatogenesis is also likely to yield a group of temporally expressed cDNA probes.

**Additional Candidates for Postmeiotic Probes.** *In vivo* and *in vitro* postmeiotic protein synthesis studies and the many morphological changes in cell shape and structure that occur during spermiogenesis indicate that many additional unique macromolecules are synthesized during spermiogenesis. Ongoing studies in many laboratories suggest proteins such as acrosin, hyaluronidase, a sperm-specific enolase, and sperm tail proteins such as the dyneins and outer dense-fiber proteins will provide additional sources of stage-specific DNA probes for this critical interval of spermatogenesis. DNA probes are also available for some protooncogenes such as *c-abl* and *c-myc* that are differentially expressed during spermatogenesis. Although *c-abl* mRNA is present in premeiotic, meiotic, and postmeiotic cell types, a novel *c-abl* mRNA of distinct size is first detected in postmeiotic cells (37). Because of its unique size, a probe specific to this shortened *c-abl* transcript could be prepared. In contrast, the protooncogene *c-myc* appears not to be expressed in testicular germ cells (38).

## Stem Cell DNA Probes

Since spermatogonia make up only a few percent of the cells found in the sexually mature mammalian testis, it has been difficult to work biochemically with this cell type, and no DNA probes unique to spermatogonia have yet been isolated for this critical stage of spermatogenesis. Because alterations in stem cell DNA will produce heritable defects, a major effort needs to be commenced to obtain an armamentarium of DNA probes specific to animal and human testicular stem cells. Recent improvements in testicular cell separation methodologies make this possible because highly enriched populations

of several types of spermatogonia can be obtained from the prepuberal testis. With poly-(A)<sup>+</sup> RNA isolated from enriched populations of spermatogonial cells, radiolabeled cDNAs can be prepared, and a differential hybridization approach similar to that previously used to obtain postmeiotic cDNAs can be conducted to isolate stem cell specific cDNAs (4). In brief, a total testis cDNA library or a cDNA library enriched with spermatogonial cDNAs would be differentially hybridized with radiolabeled cDNAs prepared from spermatogonial, meiotic, or postmeiotic cell types. The cDNAs that appear to be preferentially expressed in spermatogonia would be isolated and their temporal appearance confirmed by the early appearance of RNA in the testes of prepuberal staged mice (4). DNA sequence analysis could be used to help identify the proteins coded for by the stem cell cDNAs. One possible candidate DNA probe for a protein expressed in spermatogonia would be the DNA coding for the H2A histone stem-cell variant found in mouse embryonic spermatogenic cells (39).

## Testicular Somatic Cell DNA Probes

In addition to germ cells, the mammalian testis contains several types of specialized somatic cells such as the Sertoli, Leydig, macrophage, and peritubular cells that are needed for normal male germ-cell production. Although analysis of testicular somatic cells would require the invasive procedure of testicular biopsy, specific situations may warrant such an approach.

**Leydig Cell DNA Probes.** The endocrinology of Leydig cells has been extensively studied because of their importance in hormone production in the testis. Recently, efforts have been made to utilize DNA probes to monitor Leydig cell function. Using the technique of *in situ* hybridization, pro-opiomelanocortin (POMC) mRNAs have recently been shown to be primarily localized to Leydig cells (40). Interestingly, POMC mRNA transcripts in the testis (presumably in the Leydig cells) are approximately 600 to 800 nucleotides in length, whereas pituitary POMC mRNAs have been reported to be 1150 nucleotides. This may indicate either that differential gene expression of multiple POMC genes occurs in the testis or that there is specific mRNA processing for the POMC Leydig cell transcript. It is likely that the POMC gene(s) shows tissue-specific regulation for endorphin in the Leydig cell. Renin mRNA has also been detected by *in situ* hybridization histochemistry and immunocytochemistry in Leydig cells (41). Considering the intense biochemical attention the Leydig cell receives, the number of cell-type-specific DNA probes for Leydig cells is likely to grow rapidly.

**Sertoli Cell DNA Probes.** In addition to serving as the testicular target cell for the action of testosterone and FSH, Sertoli cells have been described as the nurse cells of the seminiferous tubule. The importance of the Sertoli cell in germ cell maturation has led to many *in vivo* and cell culture studies of this somatic testicular cell. Because of its mandatory role in germ cell development, a large number of macromolecules have been

shown either to be secreted by or to act upon the Sertoli cell. DNA probes now exist for many of these molecules, including follicle-stimulating hormone (FSH), androgen-binding protein (ABP), ceruloplasmin, transferrin, sulfated glycoproteins, inhibin, somatomedin, and the Mullerian-inhibiting substance (42-45). It is likely that this list of DNA probes will expand considerably in the coming years and provide a sizeable number of molecular markers to monitor Sertoli cell function.

## Techniques that Utilize DNA Probes to Detect the Effect of Toxic Agents

Toxic agents produce many different changes in DNA, ranging from massive deletions and translocations to point mutations. Compounds that produce large DNA lesions often inactivate genes or groups of genes essential for organ function or even the survival of the organism. Other toxic agents, however, introduce more subtle alterations such as single base-pair changes, usually at random sites throughout the genome.

When testicular function is monitored following exposure to a toxic substance, some DNA alterations are likely to directly affect spermatogenesis and fewer will lead to defects that can be localized to specific cell types with the stage-specific DNA probes. Therefore, careful study of these genes is likely to provide etiologic information on the stage of spermatogenesis at which the toxic effects are manifested. Alterations in the DNA of testicular stem cells which affect genes expressed during spermatogenesis are the most likely to be detected by hybridization analysis, since proliferation of such stem cells would produce a reasonable number of spermatozoa carrying this alteration. Some toxic agents can create heritable changes in the DNA of the testicular stem cells which will not produce any immediate phenotypic effect in the exposed individual but will be expressed in the  $F_1$  generation.

Accepting these constraints, the human male reproductive system is an excellent system to use to monitor the relative mutagenicity of compounds. On an individual by individual basis, however, these tests provide information on changes for a selected number of genes. DNA can be readily obtained from ejaculated spermatozoa, the cells that carry the paternal genome in the form that ultimately is involved in fertilization. Such studies with sperm DNA are noninvasive, inexpensive, and repeated analyses can be made from one ejaculate. Routine monitoring of individuals before workplace exposure would establish the control. Moreover, when specific perturbations to spermatogenesis are detected, repeated sampling from one individual can be accomplished over an extended period of time to establish when during the spermatogenic cycle a toxic agent has acted. For instance, the detection of an alteration in sperm DNA a few days after exposure to a toxic agent indicates that the damage occurred in the mature spermatozoa in the epididymis. The detection of changes in sperm DNA 2 weeks after exposure would indicate that

the insult occurred during spermiogenesis. Longer time intervals between exposure and detection would indicate sensitivity at earlier stages of spermatogenesis. Although testicular stem cells are often more resistant to mutation than meiotic or postmeiotic male germ cells, changes in stem cell DNA would be of more concern because stem cells are maintained throughout the life of the animal. Once an effect has been defined, animal studies can be conducted to help localize the temporal and spatial sites of action of toxic agents. Although offering a most promising approach in the future, it should be remembered that many of these assays are predicated upon our ability to monitor DNA changes in single spermatozoa, a technical problem that remains to be solved.

Despite the growing number of testicular DNA probes, the total number of such probes will always represent only a minuscule fraction of the mammalian genome. This intrinsic shortcoming of a DNA hybridization approach will be alleviated by the proposed characterization of the human genome by restriction mapping and possible sequencing of specific chromosomes or the entire human genome. Such studies will generate a vast number of useful human DNA probes and technological advances to monitor DNA.

With these considerations, several general screening techniques will be discussed. Each will use DNA isolated from ejaculated spermatozoa and will monitor for alterations in DNA by identifying differences in DNA sequence between a wild-type control and the experimentally treated sample of DNA. At first consideration, this approach appears to be seriously compromised by the presence of the many polymorphisms reported for human genes. However, detailed analysis of one of the best-studied human genes, the  $\beta$ -globin gene, has detected only a limited number (five) of polymorphisms at this locus (46). Extensive pedigree analyses for other human genes is needed to provide a better understanding of the true extent of human polymorphisms and to help establish background levels for wild-type human DNA probes.

## Restriction-Fragment-Length Polymorphisms

One popular mapping procedure uses restriction enzymes to detect DNA fragment-length polymorphisms (47). Purified genomic DNA is digested with restriction enzymes and analyzed by the Southern blot hybridization procedure with a defined cDNA or genomic probe. This relatively rapid procedure depends upon the appearance of a new DNA band or disappearance of an old DNA band to indicate an alteration in the gene sequence. It is limited by the choice of restriction enzymes, and the probe used and it is usually necessary to use many different restriction enzymes before a change is detected. If the base-pair substitution does not alter the restriction site(s) under study, the change in the sequence will not be detected.



## Hybridization with Oligonucleotides

A second DNA monitoring procedure hybridizes radiolabeled synthetic oligonucleotides that are homologous to mutant or wild-type DNAs from genomic DNA (48). Depending upon the hybridization temperature or washing conditions of the DNA duplexes, differential melting of the mismatched or perfectly paired regions of DNA can be ascertained. Since one must synthesize an oligonucleotide probe for each region monitored, this procedure would be satisfactory to screen mutations only at specific defined sites but not throughout the genome. Both the restriction-fragment-length polymorphism and oligonucleotide procedures are clearly restricted to monitoring a small number of base pairs in the genome.

## Denaturing-Gradient Gel Electrophoresis

The next two procedures using denaturing-gradient gel electrophoresis or RNA-DNA heteroduplexes are capable of screening much longer DNA fragments and, since they offer superior detection possibilities, will be described in more detail.

Denaturing gradient gel electrophoresis provides a system to separate DNA molecules by small differences in DNA sequence (49,50). Moreover, this approach does not require a close correspondence between the site of a base-pair change and a sharply defined short sequence. This method can utilize one-dimensional polyacrylamide gels containing a gradient of formamide and urea to separate duplex DNA fragments that differ by one or more base-pair mismatches.

Double-stranded DNA fragments migrate into a gel until they reach the part of the gel where the denaturant concentration is sufficient to melt the DNA. Differences in melting temperature between wild-type and duplex DNA fragments with one base-pair difference can be resolved on the gel. Although virtually all single base substitutions can be detected in cloned DNA fragments, for technical reasons, only about 25 to 40% of all possible substitutions can be detected directly in genomic DNA. Despite this limitation, this procedure clearly offers a more promising means to screen larger sections of the genome than the restriction-fragment-length polymorphism and oligonucleotide methods.

A modification of this electrophoresis procedure uses two-dimensional denaturing gradient gel electrophoresis to separate DNA fragments on the basis of length and also by the criterion of DNA sequence. Thus, DNA heteroduplexes can be distinguished by the response of their structures to gradual changes in denaturant concentration in the gel. In theory, one should be able to analyze a complex mixture of isomelting regions from one gel and obtain an analysis of each melting region by a second electrophoretic step. With additional improvements, this approach should allow large portions of a genome to be examined.

## RNA/DNA Heteroduplex Mismatches

A fourth method to detect alterations in DNA sequence is based on the hybridization of a radiolabeled RNA probe to cloned or genomic DNA (51). Mismatches are detected by the enzymatic cleavage of the single base pair mismatch of the wild-type RNA probe and the experimental DNA by RNase A. This procedure uses vector systems such as SP6 which provide a ready source of radiolabeled RNA transcripts from the DNA insert under investigation. A current limitation of this technique is the inability of RNase A or other RNases to routinely detect and cut mismatches other than C:A mismatches. This reduces the number of mismatches between the heteroduplexes that can be detected to 1 out of 12 possibilities. Although this approach is now rather limited, future modifications are likely to extend its scope.

## Pulsed Field Gel Electrophoresis

Denaturing gradient gel electrophoresis and ribonuclease cleavage of mismatches in RNA:DNA heteroduplexes both detect differences between control (wild-type) and mutant (experimentally treated) DNA sequences. Both procedures are limited by the small fraction of the genome that can be analyzed. A new technique, pulsed-field gel electrophoresis, developed to allow separation of fragments of DNA larger than can be prepared by other procedures, may overcome this problem (52,53). Genomic DNA, isolated so that random breaks are avoided, is cut into specific size fragments by addition of a rare-cutter restriction enzyme. The resulting approximately 3000 fragments average about  $2 \times 10^6$  nucleotides in length. Hybridization of DNA probes to these fragments will allow much more of the genome to be routinely examined.

## Conclusions

The application of DNA probes to monitor the effects of toxic agents on human spermatogenesis offers a novel biological marker system for the male reproductive system providing specific obstacles can be surmounted. The basic technique of molecular hybridization is highly reproducible and can readily detect DNA changes ranging in magnitude from base pair substitutions to chromosome deletions. Although hybridization techniques are relatively tedious and costly, the widespread application of this technology to problems in health and medical science suggests that vast improvements in the automation of these procedures will soon be forthcoming. The primary limitations, to date, for the application of DNA probe technology to evaluate genomic DNA alterations in germ cells are: DNA probes are available for only a very minute part of the genome; current procedures severely limit the number of probes that can be assayed at any one time, thereby restricting the percentage of the genome examined in each analysis; the



DNA from single cells, e.g., spermatozoa, cannot be analyzed. It is hoped that in the not too distant future, procedures will be developed to monitor large fragments of genomic DNA with a battery of DNA probes covering vast regions of the human genome. Advances in fluorescence detection of DNA combined with computer imaging of automated samples will aid in the analysis of DNA from single cells.

This work was supported by a National Institutes of Health Grant (GM 29224). The author thanks Jacquetta Trasler for her helpful discussions and suggestions during the preparation of this manuscript.

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